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The impact of mutations in the quorum sensing systems of *Aeromonas hydrophila*, *Vibrio anguillarum* and *Vibrio harveyi* on their virulence towards gnotobiotically cultured *Artemia franciscana*

Tom Defoirdt,[1,2] Peter Bossier,[2] Patrick Sorgeloos[2] and Willy Verstraete[1]*

[1]*Laboratory of Microbial Ecology and Technology (LabMET), Ghent University, Coupure Links 653, 9000 Gent, Belgium.*

[2]*Laboratory of Aquaculture and Artemia Reference Center, Ghent University, Rozier 44, 9000 Gent, Belgium.*

Summary

Disruption of quorum sensing, bacterial cell-to-cell communication by means of small signal molecules, has been suggested as a new anti-infective strategy for aquaculture. However, data about the impact of quorum sensing on the virulence of aquatic pathogens are scarce. In this study, a model system using gnotobiotically cultured *Artemia franciscana* was developed in order to determine the impact of mutations in the quorum sensing systems of *Aeromonas hydrophila*, *Vibrio anguillarum* and *V. harveyi* on their virulence. Mutations in the autoinducer 2 (AI-2) synthase gene *luxS*, the AI-2 receptor gene *luxP* or the response regulator gene *luxO* of the dual channel quorum sensing system of *V. harveyi* abolished virulence of the strain towards *Artemia*. Moreover, the addition of an exogenous source of AI-2 could restore the virulence of an AI-2 non-producing mutant. In contrast, none of the mutations in either the acylated homoserine lactone (AHL)-mediated component of the *V. harveyi* system or the quorum sensing systems of *Ae. hydrophila* and *V. anguillarum* had an impact on virulence of these bacteria towards *Artemia*. Our results indicate that disruption of quorum sensing could be a good alternative strategy to combat infections caused by *V. harveyi*.

Introduction

Disease outbreaks are considered to be a significant constraint to the development of the aquaculture sector (Subasinghe, 1997). Infections caused by pathogenic or opportunistic strains belonging to the species *Aeromonas hydrophila*, *Vibrio anguillarum* or *V. harveyi* cause dramatic losses in the intensive rearing of molluscs, fish and shrimp (Pass *et al*., 1987; Karunasagar *et al*., 1994; Larsen *et al*., 1994; Zhang and Austin, 2000; Muroga, 2001; Nielsen *et al*., 2001). So far, conventional antibiotics have only had limited success in the treatment of aquatic disease (Subasinghe, 1997). Moreover, their frequent use is leading to the rapid development of (multiple) resistance (Schmidt *et al*., 2000; Teo *et al*., 2000, 2002; Molina-Aja *et al*., 2002; Vivekanandhan *et al*., 2002). Therefore, there is an urgent need for alternative control techniques.

Disruption of quorum sensing, a type of cell-to-cell communication by means of small signal molecules called autoinducers, has been suggested as a new anti-infective strategy for aquaculture (Defoirdt *et al*., 2004). So far, two major quorum sensing systems have been found in Gram-negative aquatic pathogens. Like many other Gram-negative bacteria, *Ae. hydrophila* (Swift *et al*., 1997) and *V. anguillarum* (Milton *et al*., 1997) were found to contain an acylated homoserine lactone (AHL)-mediated quorum sensing system (Fig. 1A, for reviews about this type of quorum sensing see Fuqua *et al*., 2001; Miller and Bassler, 2001; Whitehead *et al*., 2001). In addition to these AHL-mediated systems, a totally different dual channel system was found in the luminescent bacterium *V. harveyi*. The first component of this system is mediated by the AHL N-(3-hydroxybutanoyl)-L-homoserine lactone (Cao and Meighen, 1989). The second component is mediated by the so-called autoinducer 2 (AI-2), which is the furanosyl borate diester 3A-methyl-5,6-dihydro-furo [2,3-D][1,3,2] dioxaborole-2,2,6,6 A tetraol (Chen *et al*., 2002). Both autoinducers are detected at the cell surface and can activate or inactivate target genes by a phosphorylation and dephosphorylation cascade (Fig. 1B; for reviews about this type of quorum sensing see McNab and Lamont, 2003; Xavier and Bassler, 2003). A dual channel quorum sensing system similar to the *V. harveyi* system was recently discovered in *V. anguillarum* (Crox-

Received 12 January, 2005; accepted 20 January, 2005. *For correspondence. E-mail Willy.Verstraete@UGent.be; Tel. (+32) 0 9264 59 76; Fax (+32) 0 9264 62 48

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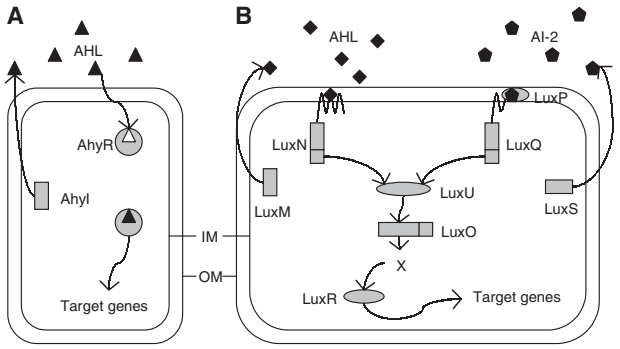

Fig. 1. Quorum sensing in *Aeromonas hydrophila*, *Vibrio anguillarum* and *Vibrio harveyi*.
A. AHL-mediated quorum sensing in *Aeromonas hydrophila* (based on Swift *et al*., 1997). AHLs are synthesized by the AhyI enzyme. At a critical AHL concentration, AHL binds to the response regulator AhyR. The AHL-AhyR complex activates transcription of target genes (e.g. exoprotease). The AhyI and AhyR homologues in *Vibrio anguillarum* are called VanI and VanR respectively (Milton *et al*., 1997).
B. The dual channel quorum sensing system of *Vibrio harveyi* (based on Henke and Bassler, 2004a). The LuxM enzyme catalyses the biosynthesis of an AHL; AI-2 biosynthesis is catalysed by the LuxS enzyme. The autoinducers are detected at the cell surface by the LuxN and LuxP-LuxQ proteins respectively. In the absence of the autoinducers, LuxN and LuxQ autophosphorylate and transfer phosphate to LuxO via LuxU. In the presence of the autoinducers, LuxN and LuxQ change from kinases to phosphatases that drain phosphate away from LuxO via LuxU. The signal is further transduced via a putative repressor, called X, and via the response regulator LuxR. LuxR activates or inactivates the transcription of target genes (e.g. the *luxCDABE* genes that are needed for bioluminescence, and the components of a type III secretion system). The LuxR, LuxM, LuxN, LuxPQ, LuxU and LuxO homologues in *Vibrio anguillarum* are called VanT, VanM, VanN, VanPQ, VanU and VanO respectively (Croxatto *et al*., 2004). IM, inner membrane; OM, outer membrane.

atto *et al*., 2004), in addition to its previously mentioned AHL-mediated system.

Several virulence-associated phenotypes in aquatic pathogens have been shown to be controlled by their quorum sensing systems. These phenotypes include biofilm formation (Croxatto *et al*., 2002; Lynch *et al*., 2002), opacity (McCarter, 1998), resistance against oxidative stress (McDougald *et al*., 2003) and the production of virulence factors such as proteases (Swift *et al*., 1997, 1999; Croxatto *et al*., 2002), haemolysin (Kim *et al*., 2003), a type III secretion system (Henke and Bassler, 2004a), extracellular toxin (Manefield *et al*., 2000) and a siderophore (Lilley and Bassler, 2000). So far, most reports dealing with quorum sensing and virulence of aquatic pathogens focused on the quorum sensing-dependent expression of one or more virulence factors. However, as pathogens usually employ a plethora of virulence factors, it is not always clear what the impact is of one single virulence factor on the virulence of the pathogen, which can be thought of as the net result of all virulence factors (Finlay and Falkow, 1997). Unfortunately, only few studies have investigated the impact of mutations in genes involved in quorum sensing or quorum sensing inhibitors on the virulence of aquatic pathogens (Milton *et al*., 1997; Milton *et al*., 2001; Croxatto *et al*., 2002, 2004; Kim *et al*., 2003; Rasch *et al*., 2004).

A major handicap in studying this type of effects is the difficulty to eliminate (or distinguish the effect of) the microbiota that is naturally present in any type of aquatic environment (Marques *et al*., 2004). Indeed, the presence of a native bacterial community might cause bias in experiments that try to determine the impact of quorum sensing mutations on virulence of pathogenic bacteria because AI-2-mediated signalling was found to be present in many different species (Xavier and Bassler, 2003). In this view, the brine shrimp *Artemia franciscana* is an excellent model organism because it can easily be cultured in gnotobiotic conditions (Verschuere *et al*., 1999). Therefore, it was used in this study in order to determine the impact of mutations in the quorum sensing systems of *Ae. hydrophila*, *V. anguillarum* and *V. harveyi* on their virulence and hence to investigate whether disruption of bacterial quorum sensing could be a valid strategy in aquaculture to combat infections caused by these important pathogens.

Results

Feed level test

Axenically hatched nauplii of *A. franciscana* were fed a 10-fold dilution series of autoclaved LVS3 bacteria in order to determine the concentration needed to obtain a survival of *Artemia* of at least 80% after 48 h. The survival of the nauplii was more or less proportional to the concentration of LVS3 (Fig. 2). The desired survival percentage was obtained with 10^7 cfu ml^{-1}. This feed level was used in all

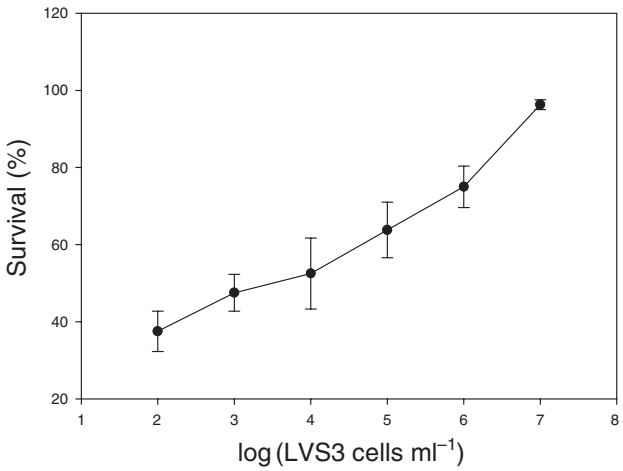

Fig. 2. Percentage survival of *Artemia* after 48 h as a function of the concentration of autoclaved LVS3 bacteria, added as a feed source (mean of 4 repeats). Error bars represent the standard error.

Table 1. Percentage survival of *Artemia* (mean ± standard error) after 48 h challenge with a non-pathogenic strain (LVS3), a known pathogen (*Vibrio campbellii* PN9801) and three strains with unknown effect to *Artemia*.

	Experiment 1		Experiment 2	
Treatment	1 × feeding	2 × feeding	1 × feeding	2 × feeding
Control	83 ± 3	89 ± 4	89 ± 2	89 ± 4
LVS3	95 ± 2	ND	96 ± 1	ND
Vibrio campbellii PN9801	4 ± 4*	16 ± 5*	6 ± 1*	16 ± 4*
Aeromonas hydrophila AH-1 N	6 ± 5*	10 ± 4*	13 ± 3*	15 ± 3*
Vibrio anguillarum NB10	6 ± 2*	9 ± 4*	14 ± 4*	15 ± 5*
Vibrio harveyi BB120	10 ± 3*	64 ± 4	35 ± 2*	78 ± 3

*Significant difference in survival between the control and the treatment of interest ($P < 0.01$).
All strains were added at 10^4 cfu ml^{-1}. *Artemia* were fed autoclaved LVS3 once (1 × feeding) at the beginning of the experiment or twice (2 × feeding); at the beginning of the experiment and after 24 h.
ND, not determined.

further experiments. The survival without the addition of LVS3 was 37 ± 1%.

Challenge tests to verify the pathogenicity of the Ae. hydrophila, V. anguillarum *and* V. harveyi *wild-type strains*

A challenge test with the wild-type *Ae. hydrophila*, *V. anguillarum* and *V. harveyi* strains was performed because it was not known whether they were pathogenic to *Artemia* or not. Live LVS3 and *V. campbellii* PN9801 were used as references of a non-pathogenic and a pathogenic strain respectively. Survival with the addition of live LVS3 bacteria was slightly (but not significantly) higher than in the controls (Table 1). *Vibrio campbellii* PN9801, *Ae. hydrophila* AH-1 N, *V. anguillarum* NB10 and *V. harveyi* BB120, in contrast, all showed to be pathogenic to *Artemia* because they were able to cause high mortality in the nauplii 48 h after addition to the culture water of *Artemia*.

We tested two different feeding regimes: feeding only once at the start of the experiment or twice, i.e. at the beginning of the experiment and after 24 h. The mortality caused by *V. campbellii* PN9801, *Ae. hydrophila* AH-1 N and *V. anguillarum* NB10 was not influenced by the feeding regime. Mortality was significant for both feeding regimes although it was slightly higher if *Artemia* was fed only at the beginning of the experiment than if it was fed twice. *Vibrio harveyi* BB120, in contrast, was significantly less virulent if *Artemia* was fed twice than if it was fed only once.

Challenge tests to determine the impact of mutations in the dual channel quorum sensing system of V. harveyi *BB120 on its virulence*

In a further series of challenge tests, we aimed at determining the effect of mutations in the dual channel quorum sensing system of *V. harveyi* on the virulence of the bacterium. The *V. harveyi* quorum sensing system consists of an AHL-mediated component and a AI-2-mediated component (Fig. 1). Mutations in the AI-2-mediated component, more precisely in the AI-2 synthase gene *luxS*, the AI-2 receptor gene *luxP* or the response regulator gene *luxO*, abolished virulence of *V. harveyi* as no significant mortality was noticed for *Artemia* challenged to the mutants MM30, BB886, MM77 and JAF548 (Table 2). Mutations in the AHL-mediated component (except for *luxO*), in contrast, had no effect on the virulence of *V. harveyi* towards *Artemia* because the mortality caused by the mutants BB152 and BB170 was similar to that caused by the wild type.

Table 2. Percentage survival of *Artemia* (mean ± standard error) after 48 h challenge with the *Vibrio harveyi* wild type and mutants with a mutation in the dual channel quorum sensing system.

Treatment	Mutation in	Experiment 1	Experiment 2
Control	–	90 ± 5	86 ± 4
Vibrio harveyi BB120	–	49 ± 4*	45 ± 4*
Vibrio harveyi BB152	*luxM* (AHL synthase)	38 ± 5*	41 ± 3*
Vibrio harveyi BB170	*luxN* (AHL receptor)	46 ± 8*	45 ± 5*
Vibrio harveyi MM30	luxS (AI-2 synthase)	81 ± 2	79 ± 2
Vibrio harveyi BB886	*luxP* (AI-2 receptor)	89 ± 3	85 ± 3
Vibrio harveyi MM77	*luxM* and *luxS*	79 ± 5	88 ± 6
Vibrio harveyi JAF548	*luxO* (AHL and AI-2 signal transduction)	76 ± 4	89 ± 4

*Significant difference in survival between the control and the treatment of interest ($P < 0.01$).
All strains were added at 10^4 cfu ml^{-1}. *Artemia* were fed autoclaved LVS3 at the start of the experiment.

Table 3. Percentage survival of *Artemia* (mean ± standard error) after 48 h challenge with the *Vibrio harveyi* AI-2-negative mutant MM30; with and without the addition of washwater of strain BB152 (as an exogenous source of AI-2).

Treatment	Experiment 1	Experiment 2
Control	89 ± 2	95 ± 2
Washwater BB152	95 ± 2	90 ± 4
Vibrio harveyi MM30	86 ± 3	85 ± 5
Vibrio harveyi MM30 + washwater BB152	50 ± 4*	59 ± 3*

*Significant difference in survival between the control and the treatment of interest ($P < 0.01$).
All strains were added at 10^4 cfu ml^{-1}. *Artemia* were fed autoclaved LVS3 at the start of the experiment.

Restoration of the virulence of the V. harveyi luxS *mutant strain MM30*

We investigated whether the addition of filtersterilized washwater of *V. harveyi* BB152 (an AHL-negative mutant) as an exogenous source of AI-2 could restore virulence of the AI-2 non-producing mutant MM30. Strain BB152 can only be luminescent if it produces AI-2 because it has a mutation in its AHL-synthase (Bassler *et al*., 1994). Therefore, luminescence of BB152 was used as a criterion for the presence of AI-2 in its washwater. We found that the BB152 washwater could restore the virulence of *V. harveyi* MM30 (Table 3). The washwater itself had no adverse effect on *Artemia* survival. Washwater of the AHL and AI-2 non-producing double mutant MM77 could not restore virulence of MM30 (data not shown).

Challenge tests to determine the impact of mutations in the AHL-mediated quorum sensing system of Ae. hydrophila *AH-1 N on its virulence*

Aeromonas hydrophila uses an AHL-mediated quorum sensing system (Fig. 1). Mortality caused by the *Ae. hydrophila* mutants with a mutation in this system was not different from that caused by the wild types (Table 4), indicating that this quorum sensing system has no impact on virulence of the bacterium towards *Artemia*.

Challenge tests to determine the impact of mutations in the AHL-mediated and dual channel quorum sensing systems of V. anguillarum NB10 on its virulence

Vibrio anguillarum contains an AHL-mediated as well as a *V. harveyi* type dual channel quorum sensing system (Fig. 1). All *V. anguillarum* mutants with a mutation in one of these systems were able to cause significant mortality in *Artemia*, although there was considerable variation (Table 5). Strains DM27 and DM63 did not cause significant mortality in the first experiment, whereas they did in

Table 4. Percentage survival of *Artemia* (mean ± standard error) after 48 h challenge with the *Aeromonas hydrophila* wild type and mutants with a mutation in the AHL-mediated quorum sensing system.

Treatment	Mutation in	Experiment 1	Experiment 2
Control	–	86 ± 4	94 ± 3
Aeromonas hydrophila AH-1 N	–	23 ± 1*	66 ± 4*
Aeromonas hydrophila AH-1N*ahyI-6*	*ahyI* (AHL synthase)	24 ± 5*	64 ± 2*
Aeromonas hydrophila AH-1N*ahyR*	*ahyR* (AHL receptor)	26 ± 2*	63 ± 3*

*Significant difference in survival between the control and the treatment of interest ($P < 0.01$).
All strains were added at 10^4 cfu ml^{-1}. *Artemia* were fed autoclaved LVS3 at the start of the experiment.

Table 5. Percentage survival of *Artemia* (mean ± standard error) after 48 h challenge with *Vibrio anguillarum* wild type and mutants in with a mutation in the AHL-mediated quorum sensing system or the dual channel quorum sensing system.

Treatment	Mutation in	Experiment 1	Experiment 2
Control	–	86 ± 4	94 ± 3
Vibrio anguillarum NB10	–	23 ± 8*	48 ± 6*
Vibrio anguillarum DM21	*vanI* (AHL synthase)	21 ± 4*	46 ± 7*
Vibrio anguillarum DM25	*vanR* (AHL receptor)	24 ± 4*	51 ± 4*
Control	–	98 ± 1	93 ± 3
Vibrio anguillarum NB10	–	57 ± 9*	30 ± 8*
Vibrio anguillarum DM27	*vanM* (AHL synthase)	68 ± 5	39 ± 9*
Vibrio anguillarum DM64	*vanN* (AHL receptor)	62 ± 4*	29 ± 6*
Vibrio anguillarum DM63	*vanQ* (AI-2 receptor)	68 ± 3	23 ± 10*
Vibrio anguillarum AC11	vanO (AHL and AI-2 signal transduction)	50 ± 4*	37 ± 4*

*Significant difference in survival between the control and the treatment of interest ($P < 0.01$).
All strains were added at 10^4 cfu ml^{-1}. *Artemia* were fed autoclaved LVS3 at the start of the experiment.

the second experiment. However, significant mortality in *Artemia* occurred after 55 h in all cases (data not shown). Therefore, none of the *V. anguillarum* quorum sensing systems has an impact on its virulence towards *Artemia*.

Discussion

In this study, a model system using gnotobiotically grown *A. franciscana* was developed in order to determine the impact of disrupting the quorum sensing systems of aquatic pathogens on their virulence. Working in the absence of other bacteria is important for this type of experiments because they might cause bias. Indeed, AI-2-mediated signalling was found to be present in many different species (Xavier and Bassler, 2003). As far as we know, it is the first time that this type of experiments was carried out in a well-defined gnotobiotic environment. To this end, sterile *Artemia* nauplii, obtained via hatching of decapsulated cysts, were cultured in filtered and autoclaved artificial seawater and fed dead LVS3 bacteria. The strain LVS3 was selected as a feed source because it was shown before to be harmless to *Artemia* (Verschuere *et al.*, 1999). The LVS3 bacteria were killed by autoclavation before addition to the *Artemia* cultures in order to eliminate any possible interactions between live LVS3 bacteria and the other strains that were added. In a first experiment, we found that a single addition of 10^7 cfu ml^{-1} of LVS3, corresponding to approximately 0.1 mg dry weight of LVS3 per mg dry weight of *Artemia*, was sufficient to obtain a survival of *Artemia* of more than 80% after 48 h (Fig. 2). This feed level was used in all further experiments.

The quorum sensing systems of *Ae. hydrophila* AH-1 N, *V. anguillarum* NB10 and *V. harveyi* BB120 have been investigated before and quorum sensing mutants have been constructed (see Table 6 for references). Therefore,

Table 6. Bacterial strains used in this study.

Strain	Relevant features	Reference
LVS3	isolate that enhances growth and survival of *Artemia*	Verschuere *et al.* (1999)
Aeromonas hydrophila strains		
AH-1 N	wild type from which strains AH-1N*ahyI-6* and AH-1N*ahyR* are derived	Swift *et al.* (1999)
AH-1N*ahyI-6*	mutation in *ahyI* (AHL synthase that produces BHL[a])	Swift *et al.* (1999)
AH-1N*ahyR*	mutation in *ahyR* (AHL receptor that detects the AHL produced by AhyI)	Swift *et al.* (1999)
Vibrio anguillarum strains		
NB10	wild type from which strains DM21, DM25, DM27, DM64, DM63 and AC11 are derived	Milton *et al.* (1997)
DM21	mutation in *vanI* (AHL synthase that produces ODHL[b] and to a lesser extend OHL[c] and OOHL[d])	Milton *et al.* (1997)
DM25	mutation in *vanR* (AHL receptor that detects the AHLs produced by VanI)	Milton *et al.* (1997)
DM27	mutation in *vanM* (AHL synthase that produces OHHHL[e] and HHL[f])	Milton *et al.* (2001)
DM64	mutation in *vanN* (AHL receptor that detects the AHLs produced by VanM)	Croxatto *et al.* (2004)
DM63	mutation in *vanQ* (AI-2 receptor)	Croxatto *et al.* (2004)
AC11	mutation in *vanO (*encoding VanO, a protein in the AHL and AI-2 signal transduction chain)	Croxatto *et al.* (2004)
Vibrio campbellii PN9801	pathogen for *Artemia*	Soto-Rodriguez *et al.* (2003)
Vibrio harveyi strains		
BB120	wild type from which strains BB152, BB170, MM30, BB886, MM77 and JAF548 are derived	Bassler *et al.* (1997)
BB152	mutation in *luxM* (AHL synthase that produces OHBHL[g])	Bassler *et al.* (1994)
BB170	mutation in *luxN* (AHL receptor that detects the AHL produced by LuxM)	Bassler *et al.* (1993)
MM30	mutation in *luxS* (AI-2 synthase)	Surette *et al.* (1999)
BB886	mutation in *luxP* (AI-2 receptor)	Bassler *et al.* (1994)
MM77	mutation in *luxM* and *luxS*	Mok *et al.* (2003)
JAF548	mutation in *luxO (*encoding LuxO, a protein in the AHL and AI-2 signal transduction chain)	Freeman and Bassler (1999)

a. N-butanoyl-L-homoserine lactone.
b. N-(3-oxodecanoyl)-L-homoserine lactone.
c. N-octanoyl-L-homoserine lactone.
d. N-(3-oxooctanoyl)-L-homoserine lactone.
e. N-(3-hydroxyhexanoyl)-L-homoserine lactone.
f. N-hexanoyl-L-homoserine lactone.
g. N-(3-hydroxybutanoyl)-L-homoserine lactone.

these strains were chosen as representatives to study the impact of disrupting the quorum sensing systems of *Ae. hydrophila*, *V. anguillarum* and *V. harveyi* on their virulence towards a higher organism. A first challenge showed that the wild-type strains were pathogenic to *Artemia* as they were able to cause high mortality if they were added in a concentration as low as 10^4 cfu ml^{-1} to the *Artemia* culture water (Table 1). We tested two different feeding regimes in order to get an idea about the virulence of the strains. The virulence of *Ae. hydrophila* AH-1 N and *V. anguillarum* NB10 was not affected by the feeding regime, whereas *V. harveyi* BB120 was considerably less virulent if *Artemia* was fed daily than if it was fed only once. A high survival of the nauplii (as occurred in the controls if LVS3 was added only once at the beginning of the experiment) does not exclude that they are underfed to a certain degree. It would mean that *V. harveyi* BB120 only caused significant mortality if *Artemia* experienced stress resulting from underfeeding, indicating that the strain is an opportunistic pathogen.

A further series of challenge tests revealed that mutations in the AI-2-mediated component of the dual channel quorum sensing system of *V. harveyi* BB120 abolished virulence of the strain, whereas mutations in the AHL-mediated component had no effect on its virulence (Table 2). Furthermore, we investigated whether an exogenous source of AI-2 could restore the virulence of the AI-2-negative mutant MM30. Filtersterilized washwater of a *V. harveyi* BB152 culture was chosen as an exogenous source of AI-2 because it is not yet possible to obtain the molecule in a purified form (B. Bassler, pers. comm.). Strain BB152 can only be luminescent if it produces AI-2 because it has a mutation in its AHL-synthase (Bassler *et al*., 1994). Consequently, luminescence of the strain was used as a criterion for the presence of AI-2 in its washwater. The BB152 washwater could restore the virulence of *V. harveyi* MM30 (Table 3).

Our results show that *V. harveyi* can distinguish between AHL and AI-2. Mok and colleagues (2003) proposed two explanations for such a pattern of regulation: there could be a difference in the kinase-to-phosphatase ratio of the AHL receptor compared with the AI-2 receptor or there could be an additional signal transduction loop (in addition to the system as drawn in Fig. 1). Interestingly, at submission of this article, Henke and Bassler (2004b) reported a third component in the quorum sensing system of *V. harveyi*. The new component is mediated by the so-called *cholerae* autoinducer 1 (CAI-1), which structure is unknown. The autoinducer is synthesized by the CqsA protein and detected by the CqsS protein. Like the two components as drawn in Fig. 1, the signal is transduced via LuxU and LuxO. In our opinion, however, an additional signal transduction loop is not a probable explanation for the regulation of virulence in our experimental conditions. Indeed, the *luxO* mutant JAF548, in which LuxO is fixed in the low cell density state (and hence is not responsive anymore to any of the autoinducers), was not virulent to *Artemia*. Therefore, quorum sensing-regulated expression of virulence must be transduced via LuxO and not via any additional signal transduction loop. Moreover, the mortality caused by the LuxO mutant was similar to that caused by the mutants with a mutation in the AI-2-mediated component of the *V. harveyi* quorum sensing system, indicating that the influence of the other components is not significant. A difference in the kinase-to-phosphatase ratio of the receptors remains possible. Interestingly, Henke and Bassler (2004b) mentioned that the relative kinase-to-phosphatase ratio of the receptors is different in different environmental conditions. They found that the relative signal strength for bioluminescence induction was AHL > AI-2 > CAI-1 in liquid medium, whereas it was AHL > CAI-1 > AI-2 on agar plates. Under the *in vivo* conditions in our experiments, in contrast, the AI-2 signal appeared to be the strongest signal.

It has been shown before that blocking the AHL-mediated quorum sensing system of *Ae. hydrophila* AH-1 N with signal molecule analogues decreased exoprotease production by the bacterium (Swift *et al*., 1999). However, we found that mortality of *Artemia* caused by the same *Ae. hydrophila* strain was not different from that caused by the mutants with a mutation in the AHL-mediated quorum sensing system of the bacterium (Table 4). These results indicate that a decrease in the expression of a virulence factor does not necessarily imply a decreased virulence, emphasizing once more the importance of *in vivo* experiments as performed in this study.

We found that neither mutations in the AHL-mediated system nor in the dual channel system of *V. anguillarum* NB10 had any effect on its virulence towards *Artemia* (Table 5). Our data are in accordance with results obtained with rainbow trout (*Oncorhynchus mykiss*) challenged to the same *V. anguillarum* wild type and mutant strains as used in this study (Milton *et al*., 1997; Milton *et al*., 2001; Croxatto *et al*., 2002). In contrast, Rasch and colleagues (2004) found that quorum sensing antagonistic furanones could protect rainbow trout from vibriosis caused by *V. anguillarum*. A possible explanation for the contradiction between these results might be that both quorum sensing systems of *V. anguillarum* can activate its virulence. Therefore, it would be necessary to block both systems in order to decrease virulence of the bacterium, which was probably the case in the research of Rasch and colleagues (2004). Indeed, it has been shown before that quorum sensing antagonistic furanones block AHL-mediated systems as well as the *V. harveyi* type dual channel system (Givskov *et al*., 1996; McDougald *et al*., 2003). Other explanations could be that apart from quorum sensing blockade, the furanone has activity

towards other pathways in *V. anguillarum* or that it activates the immune system of rainbow trout (Rasch *et al*., 2004).

All together, our results indicate that disruption of quorum sensing could be a good alternative strategy to combat infections caused by *V. harveyi*. *Vibrio harveyi* is an important pathogen in the intensive rearing of molluscs (Pass *et al*., 1987), finfish (Zhang and Austin, 2000) and especially shrimp (Karunasagar *et al*., 1994). Moreover, several pathogenic *V. harveyi* strains are found to be resistant against antibiotics (Teo *et al*., 2000, 2002) and peroxide (Vattanaviboon and Mongkolsuk, 2001). Therefore, disruption of the AI-2-mediated component of the *V. harveyi* quorum sensing system might prove to be a good additional tool to combat infections caused by this pathogen.

Experimental procedures

Bacterial strains and growth conditions

The bacterial strains that were used in this study are described in Table 6. All bacterial strains were stored in 40% glycerol at −80°C. Ten microlitres of these stored cultures were inoculated into fresh Marine Broth (Difco Laboratories, Detroit, MI, USA) and incubated for 24 h at 28°C under constant agitation (100 min^{-1}). Bacterial cell numbers were determined by plating dilution series in sterile physiological solution (8.5 g NaCl l^{-1}) on Marine Agar (Difco) and incubating for 24 h at 28°C.

Axenic hatching and culture of Artemia

All experiments were performed with high quality hatching cysts of *A. franciscana* (EG® Type, batch 6940, INVE Aquaculture, Baasrode, Belgium). Two hundred milligrams of cysts were hydrated in 18 ml of tap water during 1 h. Sterile cysts and nauplii were obtained via decapsulation, adapted from Marques and colleagues (2004). Briefly, 660 μl of NaOH (32%) and 10 ml of NaOCl (50%) were added to the hydrated cyst suspension. The decapsulation was stopped after 2 min by adding 14 ml of $Na_2S_2O_3$ (10 g l^{-1}). During the reaction, 0.22 μm filtered aeration was provided. The decapsulated cysts were washed with filtered (0.22 μm) and autoclaved artificial seawater containing 35 g l^{-1} of Instant Ocean synthetic sea salt (Aquarium Systems, Sarrebourg, France). The cysts were resuspended in a 50-ml tube containing 30 ml of filtered and autoclaved artificial seawater and hatched for 24 h on a rotor (4 min^{-1}) at 28°C with constant illumination (approximately 2000 lux). After hatching, groups of 20 nauplii were transferred to new sterile 50-ml tubes that contained 30 ml of filtered and autoclaved artificial seawater. The nauplii were fed and a bacterial strain of interest was added. Finally, the tubes were put back on the rotor and kept at 28°C. All manipulations were performed under a laminar flow hood in order to maintain sterility of the cysts and nauplii.

Feeding of Artemia *– feed level test*

Artemia were fed autoclaved LVS3 bacteria, grown in Marine Broth as described above. Before autoclaving, LVS3 cells were washed twice with filtered and autoclaved artificial seawater. In order to determine the concentration of LVS3 bacteria needed to obtain a survival of *Artemia* of at least 80% after 48 h, the *Artemia* nauplii were fed a 10-fold dilution series of a LVS3 culture. The final concentration in the *Artemia* culture water was 0, 10^2, 10^3, 10^4, 10^5, 10^6 and 10^7 cells ml^{-1} respectively. Each treatment was carried out in quadruplicate. The survival of *Artemia* was scored 48 h after the addition of LVS3.

Challenge tests

After incubation, bacterial cells were washed twice in filtered and autoclaved artificial seawater. The washed cells were stored for 1 day at 4°C before addition to *Artemia* cultures, 24 h after the start of *Artemia* hatching. The bacterial density used in all challenge tests was 10^4 cfu ml^{-1} of *Artemia* culture water. Autoclaved LVS3 bacteria were added once at the start of the challenge test (unless otherwise stated). *Artemia* cultures to which only autoclaved LVS3 bacteria were added were used as controls. The survival of *Artemia* was scored 48 h after the addition of the strains. Each treatment was carried out in quadruplicate and each experiment was repeated twice. The aim of repeating each experiment twice (and of reporting both results) is to investigate whether (and to demonstrate that) differences between different treatments are reproducible, although the absolute values might be different because of the fact that each repeat was performed with a different batch of nauplii.

Methods used to verify axenicity

In each test, the sterility of the feed and the control treatments (to which no live bacteria were added) were checked at the end of the challenge by mixing 1 ml of the feed suspension or *Artemia* culture water with 9 ml of fresh Marine Broth in a test tube and incubating the mixture for 2 days at 28°C. If a control tube would have been found to be contaminated (as manifested by increased turbidity of the Marine Broth), the data form the corresponding experiment would not have been considered and the experiment would have been repeated.

Preparation of washwater of V. harveyi *strain BB152*

Vibrio harveyi BB152 was grown as described above. After incubation, the culture was centrifuged for 10 min at 2000 *g* and the cells were resuspended in filtered and autoclaved artificial seawater. The cells were centrifuged once more after 15 min of incubation and the supernatant was filter sterilized over 0.22-μm Millipore filters (Millipore, Bedford, USA). Three millilitres of the washwater was added to 27 ml of *Artemia* culture water.

Statistics

For each experiment, the mean survival for each treatment was compared with the mean survival in the control by an independent samples *t*-test, using the SPSS software, version

12.0. Mortality caused by a strain was considered significant if the *P*-value of the *t*-test was below 0.01.

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